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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of

Applicant : PAVESIO Alessandra et al.
Serial No. : 09/700,142
Filed : November 9, 2000
Title : BIOMATERIALS CONTAINING HYALURONIC ACID DERIVATIVES IN THE FORM OF THREE-DIMENSIONAL STRUCTURES FREE FROM CELLULAR COMPONENTS OR PRODUCTS THEREOF FOR THE IN VIVO REGENERATION OF TISSUE CELLS
Examiner : Dwayne C. Jones
Art Unit : 1614

DECLARATION UNDER CFR 1.132

I, Giovanni Abatangelo, being duly sworn depose and say that:

1. I am an Italian citizen residing in Padova, Italy.

2. I am familiar with the English language.

3. I further declare that:

A) Universities or Colleges attended:

I graduated in Medicine in the academic year 1965 at the University of Padua, Italy.

In 1977 I completed the Postdoctoral Specialization in Clinical Pathology at the University of Padua.

B) Publications

I am author and co-author of 50 scientific papers on peer reviewed journals.

C) Professional experiences and research activity

From 1965 to 1967 I worked as a Postdoctoral Fellow in the Department of Biochemistry of the Baylor University in Houston, Texas, USA.

In academic year 1970 I became Lecturer at the Institute of Histology of the University of Padua, and in 1975 I was promoted to Full Professor of Histology-Embriology.

Presently, I am still working as Full Professor of Histology-Embriology at the University of Padua, and researcher. During the last decade my research activity is been mainly devoted to tissue engineering of skin and cartilage.

Furthermore, I am:

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- President of the Italian Society of Cutaneous Biology and Member of the Editorial Board of "Wound Repair and Regeneration" (Mosby Inc., St. Louis, MO, USA);
- Organizer and President of the 1° and 2° International Symposia on Cutaneous Development, Aging and Repair (Padua, Oct. 4-7 1987 and Oct. 13-16 1991), and of the 5° Annual Meeting of the European Tissue Repair Society;
- President of the Consortium "TissueTech", a non-profit joint company between University of Padua and Biotechnological Industries, dealing with research projects in biomaterials and tissue engineering for clinical use, supported by the Italian Government;
- Coordinator of the research project "Biomaterials in Orthopedics: Scaffolds for Bio-engineered Cartilage", funded by Italian Ministry of University and Research and Italian National Council Research;
- Research Leader of a 4-year Grant from the European Community entitled "Peripheral Nerve Repair".

4. I further declare that the following Experiments 1-3 were carried out under my direct responsibility and supervision, in order to demonstrate the ability in regeneration of tissues of the biomaterials of the invention containing hyaluronic acid derivatives, free from cellular components and/or products thereof and processed in the form of a three-dimensional structure enclosing hollow spaces formed by communicating pores or in the form of fine fibers worked into the form of a non-woven fabric, in this case the biomaterial consisting of hyaluronic acid benzyl ester having an esterification degree of 65%.

EXPERIMENT 1

Fifteen black and white male rats from Lister of Liverpool, weighing between 300 and 350 g, were used. The animals were anaesthetized with immunoglobulin (C-Vet Ltd, Bury St. Edmunds, UK) at a dose of 0.1 ml per 100 g of body weight. The area to be operated on was shaved and the skin cleansed, the femur was exposed by the cut and drain method.

A hole with a diameter of 1.76 mm was drilled in the bones of both paws under abundant irrigation with saline solution.

A periosteal support was fitted under the bone to protect the underlying muscle.

Having inserted the implants in the lesion on a level with the surface of the bone, the periosteum and the soft tissue were replaced over the surface of the wound and stitched. The outer wound was sealed with a coating of acrylic spray (Nobecutane®, Astra

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Pharmaceutica, Kings Langley, UK) to reduce the risk of infection. The animals were revived with Revivon® (Reckitt & Coleman) at a dose of 0.05 ml per 100 g of body weight. The animals were treated as reported in the Table 1 below.

Table 1

No. of animals	Groups	Treatment
3	1	HA (powder)
3	2	HYAFF11 (non-woven fabric)
3	3	HYAFF11p80 (non-woven fabric)
3	4	HYAFF11p65 (non-woven fabric)
3	5	Control

In the Table 1 above reported, the following abbreviations are used:

HA: hyaluronic acid fractions hyalastine® and hyalectin® (EP 0138572);

HYAFF11 (non-woven fabric): benzyl ester of hyaluronic acid with 100% esterification in the form of fine fibres worked into the form of a non-woven fabric (US 5,520,916);

HYAFF11p65 (non-woven fabric): benzyl ester of hyaluronic acid with 65% esterification in the form of fine fibres worked into the form of a non-woven fabric (US 5,520,916);

HYAFF11p80 (non-woven fabric): benzyl ester of hyaluornic acid with 80% esterification in the form of fine fibres worked into the form of a non-woven fabric (US 5,520,916).

Four weeks later the animals were sacrificed by the standard method. Their 10 femurs were removed and cut so as to separate the treated part. The bone marrow was removed by carefully injecting 40% methanol, taking care not to damage the part containing the implant.

The samples were fixed and decalcified with a Lillie solution for a minimum of three days, washed for two days in 75% alcohol, dehydrated by washing with is alcohol at increasing percentages and, lastly, embedded in paraffin. The samples were then sectioned perpendicularly to the bone surface and parallel to the diameter of the defect, obtaining sections with a thickness of 7 μm.

The four most central sections of each sample were stained using haematotoxylin-eosin staining and Mallory's triple stain.

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Histomorphometric assessment was performed using a microscope for image analysis (IBAS).

The percentage of bone regeneration after 24 days is shown in Graph 1 herewith enclosed. As can be seen from the graph, the benzyl ester of hyaluronic acid with 65% esterification in the form of fine fibers worked into the form of a non-woven fabric induces, surprisingly a greater degree of bone regeneration than the total benzyl ester made into the same form, and hyaluronic acid in powder form.

EXPERIMENT 2

Six male, Yucatan micropigs of about one year old and weighing about 25-30 kg were used.

The animals were anaesthetized throughout the experiment by intramuscular injection with 8 ml of a solution containing Xilazine® (20 mg/ml) and Ketamine® (50 mg/ml) in a ratio of 1:2. Anaesthesia was boosted when necessary by intravenous injections of 5-10 mg/kg of a solution of pentobarbital at a concentration of 60 mg/ml. Each animal was shaved while under anaesthetic. The shaved part was first washed with surgical detergent and then disinfected with an iodine solution. Six circular wounds (3 per side), measuring 4 cm in diameter were performed, completely removing both the dermal tissue and epidermal tissue while leaving the underlying muscle fascia intact.

A chamber of PTFE was placed on the wound bed using the edges of skin to fix the base of the chamber at a subcutaneous level. The chamber was closed with 2/0 silk suture sewn to the surrounding skin.

The benzyl esters of hyaluronic acid with 65% and 100% esterification in the form of fine fibers processed in the form of non-woven tissue, were applied to the wounds as shown below in Table 2.

Table 2

Groups	**No. of sites tested**	**Treatment**	**Dose**	**Administration route**
1	12	HYAFF11p65 (non-woven fabric)**	5x5 cm	Topical
2	12	HYAFF11 (non-woven fabric)**	5x5 cm	Topical
3	12	---	---	---

** applications performed on the day of surgery and on the following 7th, 14th, 21st and 28th days.

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In the Table 2 above reported, the following abbreviations are used:

HYAFF11p65 (non-woven fabric): benzyl ester of hyaluronic acid with 65% esterification in the form of fine fibers worked into the form of a non-woven fabric (US 5,520,916);

HYAFF11 (non-woven fabric): benzyl ester of hyaluronic acid with 100% esterification in the form of fine fibers worked into the form of a non-woven fabric (US 5,520,916).

After treatment, the wounds were covered with vaseline-soaked gauzes and the animals were wrapped in elastic bandages. In order to protect the wounds, lightweight, stiff jackets were tied round the animals' trunks.

The percentage of re-epithelialization was calculated using a microscope for image analysis (IBAS).

In Graph 2 herewith enclosed the percentage of re-epithelialization per treatment is showed.

As can be seen from Graph 2, the wounds treated with HYAFF11 p65 in the form of fine fibers made into non-woven fabrics present surprisingly high re-epithelialization results compared with those treated with the total ester made into the same form.

As the epithelial tissue cells could not possibly have come from either hair follicles, because the wound was a full-thickness wound, or from the edges of the wound, because they had been separated from the treatment site by the chamber of PTFE, the epithelium which formed in the presence of the test material must have originated from the progenitor cells from the wound bed recruited by the biomaterial.

EXPERIMENT 3

The aim of this study is to test the biomaterials in the form of three-dimensional structures enclosing hollow spaces formed by communicating pores constituted by hyaluronic acid esters as materials to use as implants for the treatment of osteochondral defects in rabbit.

Twenty-four 4-months-old New Zealand White rabbits weighing 2.6-3.0 Kg were anesthetized, then their knee joint was exposed through a medial parapatellar longitudinal incision; the capsule was incised and the medial femoral condyle was exposed after lateral luxation of the patella and resection of the infrapatellar fat tissue. With the knee maximally flexed, a full thickness defect (3 mm diameter by 3 mm deep) through articular cartilage and into subchondral bone was prepared on the center of the condyle. The implants made of HYAFF 11 (total benzyl ester of hyaluronic acid) in the form of three-

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dimensional structures enclosing hollow spaces formed by communicating pores, were then placed into 24 defects created as above said, and 24 defects were left untreated.

The rabbits were sacrificed either 4 or 12 weeks after surgery and the condyles were subjected to histological analysis, and the performance of the test materials was established by semi-quantitative grading of the histological sections based on a 29-point modification of the O'Driscoll's 24-point scale.

Four weeks after surgery, the majority of the untreated defects were filled with bone tissue to the level of the tidemark, whereas the noncalcified top layer varied from undifferentiated fibrous and fibrocartilagenous tissue to hyaline-like tissue. At four weeks, the defects treated with HYAFF11 presented a rim of chondrogenic cells at the interface between the defect and the host tissue, while the central part of the defect had low cell density with very little or no matrix. The top layer varied: it was composed of either hyaline-like cartilage or fibrocartilage.

At twelve weeks, the untreated defects presented bone filling beyond the level of the tidemark. Some specimens had a thin, non-calcified top layer composed of varying amounts of fibrous tissue and fibrocartilage, whereas other specimens had a thick nonmineralised layer composed of fibrocartilage with various degrees of fibrillation. None of the untreated defects healed completely with hyaline cartilage.

Twelve weeks after surgery, most of the HYAFF11-treated defects exhibited bone fill. Less than 25% of the defects had an area in the center of the implant that was either unfilled or filled with undifferentiated fibrous tissue. The HYAFF11 material that was in the specimens at four weeks was completely absent at twelve weeks.

The points scored by the specimens at 12 weeks are shown below in Table 3, and reported as median value (range):

Table 3

Group	Score	No. of sites tested
Non-treated	13.8 (5.0-18.3)	12
HYAFF11	17.1 (13.3-22.0)	12

In the following Table 4 the histologic scores as median value (range) are reported for the most significant categories of the scoring system for the twelve-weeks specimens:

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Table 4

Category	Non-treated	HYAFF11
% hyaline articular cartilage	1.50 (0.0-6.0)	5.75 (3.5-8.0)
thickness	0.63 (0.0-1.5)	1.00 (0.8-1.5)

From the results in the above Table 4, it may be observed that the scores of the histologic tests at twelve weeks for the defects treated with HYAFF11 were significantly higher than those for untreated defects ($p<0.05$). Defects treated with HYAFF11 consistently had better scores than untreated defects for both the percentage of hyaline cartilage and the thickness of the cartilagineous layer within the repair site.

The study illustrated above has proved that the biomaterials made of hyaluronic acid esters processed in the form of a three-dimensional structure enclosing hollow spaces formed by communicating pores, facilitate the natural wound-healing response, creating an environment which is very similar to the embryonic one, wherein the endogenous progenitor cells can regenerate damaged tissue.

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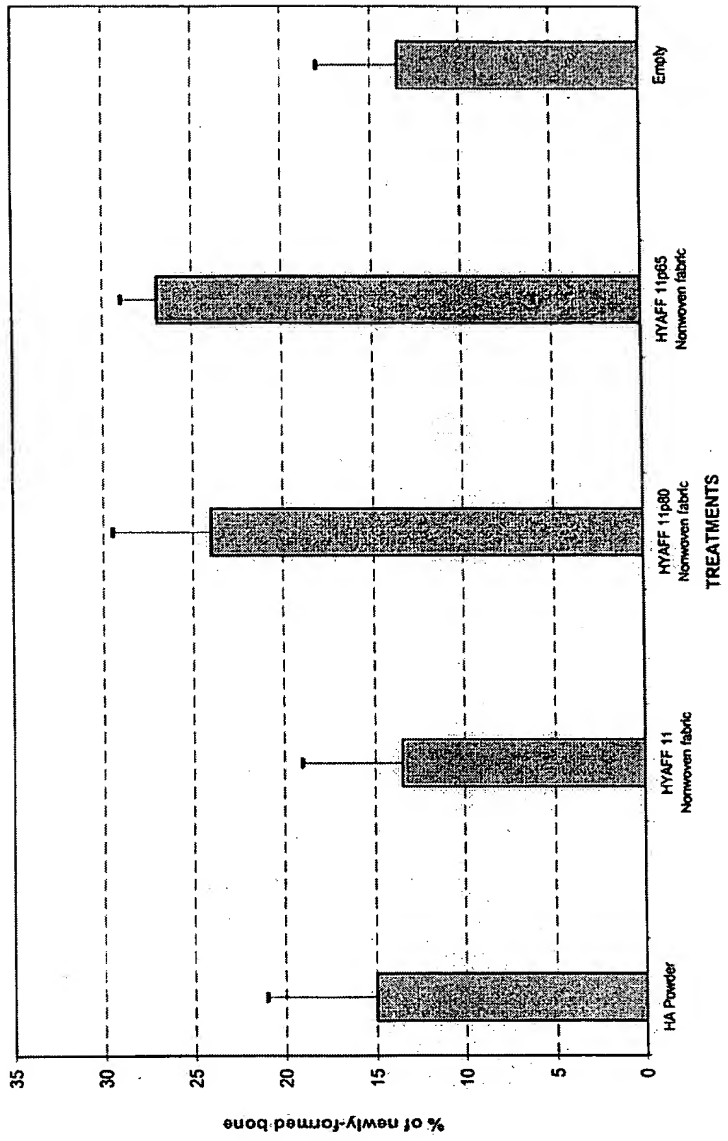
Graph 1



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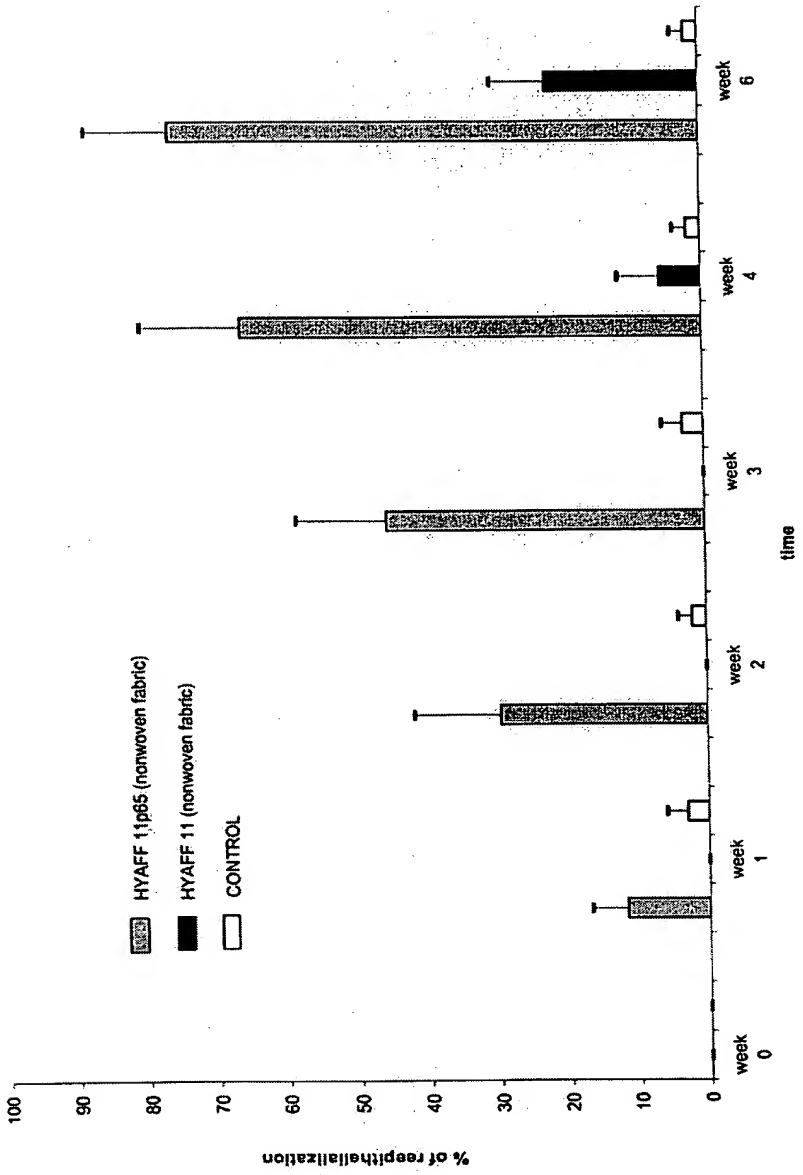
Graph 2



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4. I finally declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that such willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the applications or any patents or re-examination certificate issued thereon.

Abano Terme, (2004-06-17)

Giovanni Abatangelo